Synapse Formation by Neuroblastoma Hybrid Cells

M. NIRENBERG, S.P. WILSON, H. HIGASHIDA, A. ROTTER, K. KREUGER, N. BUSIS, R. RAY, J. KENIMER, M. ADLER, AND H. FUKUI

Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

The most widely held hypothesis concerning the mechanism of synapse formation during the last 20 years has been the chemoaffinity hypothesis of Sperry (1963), i.e., that neurons distinguish appropriate from inappropriate synaptic partner cells by interactions between molecules that code for cell recognition. Although Sperry avoided detailed models, many investigators have assumed that neurons or other cells that possess different cell-recognition molecules are generated and that the cells then sort out in appropriate sequences by differential adhesiveness before synaptic connections form. Another quite different hypothesis is that neurons may form relatively stable intercellular attachments during differentiation and that environmental factors and/or transynaptic signals may regulate the expression of genes and thereby determine the final pathway for differentiation and the type of neuron and synapse to be expressed. These hypotheses are not mutually exclusive; both may be involved in the assembly of synaptic circuits.

We have established clonal lines of neuroblastoma and somatic hybrid cells that express neural properties and form synapses with cultured striated muscle cells, and we have used the cells as model systems for studies on the effects of extracellular molecules on the expression of neural properties and on synaptogenesis. We find that the expression of stimulus-secretion coupling and synaptogenesis can be regulated in neuroblastoma hybrid cells by receptor-mediated events that are coupled to the activation of adenylate cyclase. We also find that many ^{35}S-labeled glycoproteins are affected when cells are shifted to a synapse competent state.

RESULTS

Synapse Competent and Defective Cell Lines

Twenty-six neuroblastoma or hybrid cell lines were tested for their ability to synthesize and release acetylcholine (ACh) and form synapses with cultured rat striated muscle cells (Higashida et al. 1978; Wilson et al. 1978). Synapses were detected by intracellular recording of miniature end-plate potentials and evoked muscle responses. Photomicrographs of cells from four of the five cell lines found that form abundant synapses are shown in Figure 1. NBr10-A and NBr20-A cells originated by fusion of mouse neuroblastoma N18TG-2 (Minna et al. 1972) with clonal BRL-30E rat liver cells; NCB-20, by fusion of N18TG- 2 cells with fetal Chinese hamster brain cells; and NG108-15 (Nelson et al. 1976), by fusion of N18TG-2 with C6BU-1 rat glioma cells (Amano et al. 1974). Muscle responses to ACh secreted by the neural cells were inhibited reversibly by 5 μM *d*-tubocurarine and irreversibly by 10 nM α-bungarotoxin. Omission of Ca^{++} ions in the medium reduced the frequency of miniature end-plate potentials (Nelson et al. 1978).

The expression of some neural properties by the cells is dependent on prolonged elevation of cellular cAMP. For example, untreated cells have few or no neurites and relatively small soma. However, treatment of cells for 7 days with 1 mM dibutyryl cAMP promotes neurite extension, and decreasing the concentration of fetal bovine serum reduces neurite retraction (Seeds et al. 1970). Neurites are produced in profusion under these conditions as shown in Figure 1.

Other cell lines were found that synthesize ACh and adhere well to myotubes, but do not form synapses. The phenotypes of the cell lines are summarized in Table 1. Cells from five lines synthesize ACh, take up Ca^{++} ions from the medium and secrete ACh when depolarized by 80 mM K^+ ions, possess small clear vesicles 60 nm in diameter and large dense-core vesicles 180 nm in diameter, synthesize and secrete a protein that stimulates the aggregation of nicotinic ACh receptors (AChR) on myotube plasma membranes, and form many synapses with cultured myotubes. Cells from three lines take up Ca^{++} ions slowly and to only a small extent, secrete relatively little ACh when depolarized, and form few synapses with muscle cells. Two cell lines lack functional voltage-sensitive Ca^{++} channels and therefore do not take up $^{45}Ca^{++}$ or secrete more ACh when depolarized and do not form synapses. Cells from five lines take up Ca^{++} when depolarized, but do not respond to the increase in cytoplasmic Ca^{++} concentration by secreting ACh, and form few or no synapses. These cells release ACh into the medium in the basal unstimulated state but lack a Ca^{++}-dependent ACh secretion reaction(s). Three cell lines possess small clear vesicles, but lack both large dense-core vesicles and functional AChR aggregation protein, and form few or no synapses. Nine additional cell lines have little or no choline acetyltransferase activity and thus synthesize little or no ACh and do not form synapses with striated muscle cells.

Regulation of Synaptogenesis

Neuroblastoma and hybrid cells have PGE_1 receptors that mediate the activation of adenylate cyclase (Sharma

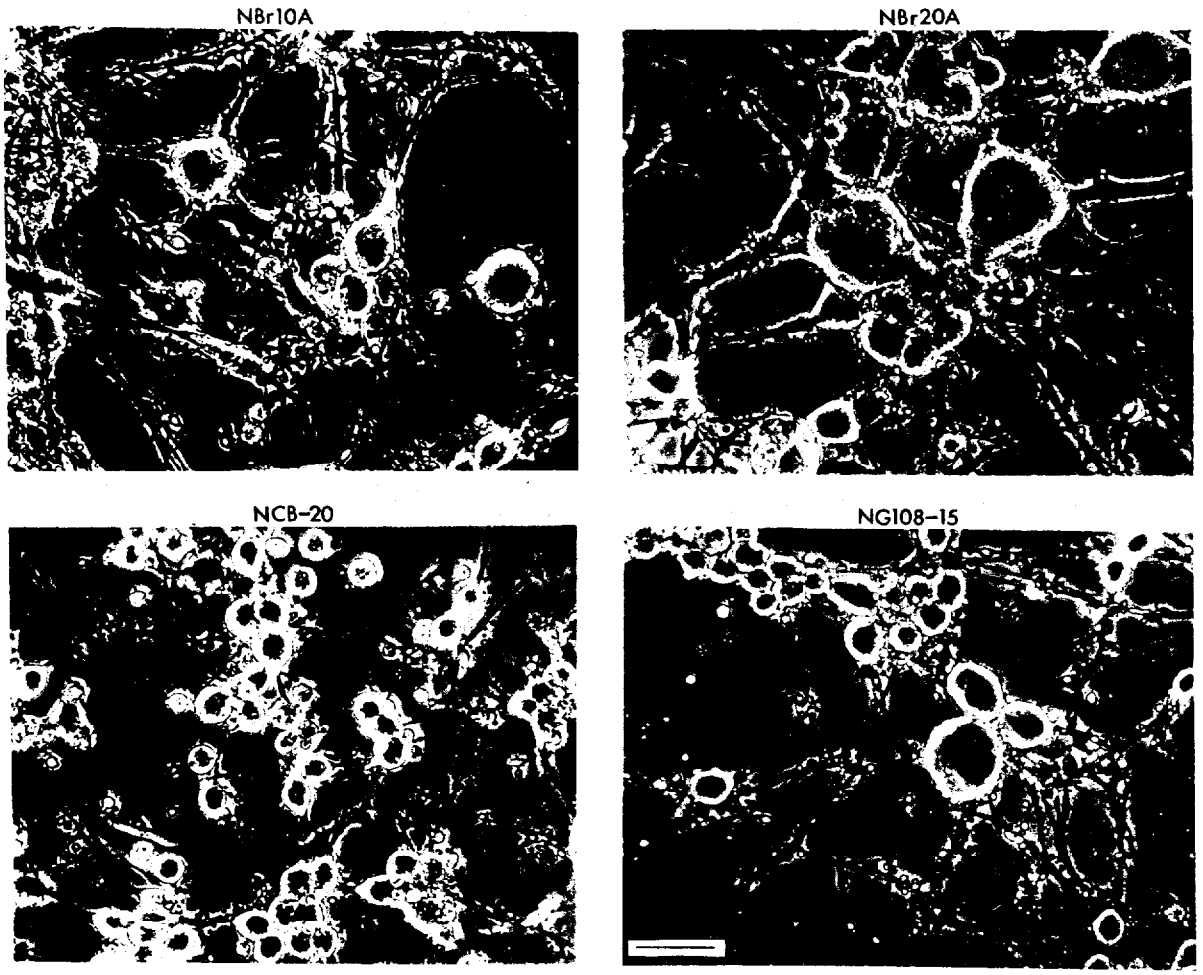

Figure 1. Neuroblastoma hybrid cells from lines that form many synapses with cultured myotubes were treated for 7 days with 1 mM dibutyryl cAMP, and the concentration of fetal bovine serum was reduced from 5% to 1% between the fifth and seventh days (H. Higashida et al., in prep.). Bar, 50 μm.

et al. 1975b, 1977). Cells were cultured in the presence of PGE_1 to increase the endogenous rate of synthesis of cAMP and elevate intracellular levels of cAMP (Matsuzawa and Nirenberg 1975; Sharma et al. 1975a). Treatment of cells with PGE_1 has no immediate effect on cell membrane potential or rate of ACh secretion (McGee et al. 1978).

In Table 2 are shown the effects of treating NG108-15 cells and rat myotubes with PGE_1, or a cyclic nucleotide phosphodiesterase inhibitor such as dibutyryl cAMP or theophylline, on the percentage of myotubes tested that were innervated and the frequency of miniature end-plate potentials of myotubes. The cells were cocultured and treated with the compounds shown in Table 2 for 5–7 days before myotubes were assayed for synapses by intracellular microelectrode recording. Treatment of cells with 1 mM dibutyryl cAMP, 1 mM theophylline, or 10 μM PGE_1 increased the percentage of muscle cells tested that were innervated from 15% to approximately 60% and also

Table 1. Cell Line Phenotypes

Cell lines	ACh formation	K^+-dependent		Vesicles		AChR aggregation protein	Synapse
		$^{45}Ca^{++}$ uptake	[^{3}H]ACh release	small clear	large dense core		
5	+	+ + +	+ + +	+	+	+	+ + +
3	+	+	+	+	+	+	+
2	+	−	−	+	+	+	−
5	+	+ +	−	+	+	+	− or +
3	+	+ +	±	+	−	−	− or +
9	−		−				−

Data from Higashida et al. (1978), Rotter et al. (1978), Wilson et al. (1978), and N.A. Busis, M.P. Daniels, H.C. Bauer, P.A. Pudimat, P. Sonderegger, A.E. Schaffner, and M. Nirenberg.

Table 2. Effect of Culture Conditions on Synaptogenesis and ACh Secretion by NG108-15 Cells

Culture conditions	% myotubes with synapses	Synaptic responses/ min/ myotube
Control	15	0.7
Dibutyryl cAMP (1 mM)	55	14
Theophylline (1 mM)	64	10
PGE_1 (10 μM)	63	11
PGE_1 (10 μM) + theophylline (1 mM)	98	32

Each value is the mean of values obtained from more than 75 myotubes (Higashida et al. 1978).

resulted in 14- to 20-fold increases in the mean miniature end-plate-potential frequencies (Higashida et al. 1978). Presumably, each miniature end-plate potential reflects the response of a myotube to the spontaneous release of ACh from a single NG108-15 vesicle. The effects of PGE_1 and theophylline were additive or synergistic since 98% of the myotubes tested were innervated and a 45-fold increase in the miniature end-plate-potential frequency was observed.

As shown in Figure 2, the effects of PGE_1, theophylline, or dibutyryl cAMP on synaptogenesis are expressed slowly. Half-maximal increases in myotube innervation required 1–2 days of treatment. In other experiments not shown here, cells were incubated with PGE_1, theophylline, dibutyryl cAMP, or PGE_1 and theophylline for 5–7 days; the cells were then incubated for an additional 4–14 days in the absence of the compounds to determine whether the effects on synapses were reversible. On cessation of treatment, synapses and ACh secretion rates slowly returned to control values over a period of 7–10 days. Thus, the effects of the compounds on synaptogenesis were expressed slowly, and on withdrawal of the compounds, the effects were reversed slowly. Treatment of NG108-15 cells with PGE_1 and Ro20-1724, a cAMP phosphodiesterase inhibitor, also increased the number of synapses. However, no reversal was detected due to withdrawal of these compounds.

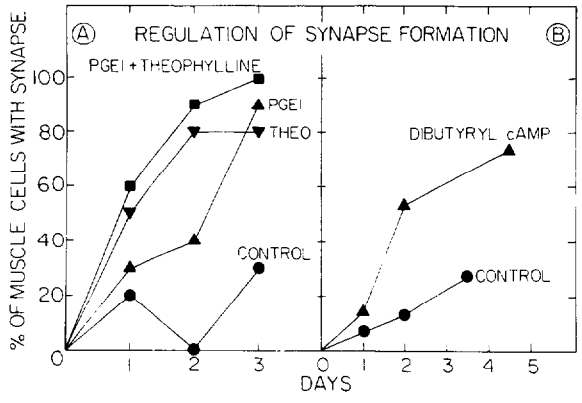

Figure 2. Effects of PGE_1, theophylline, or dibutyryl cAMP on the percentage of myotubes innervated by NG108-15 cells are shown as a function of time of coculture. At zero time, 2×10^5 mechanically dissociated NG108-15 cells were added to each 35-mm petri dish, which contained well-differentiated myotubes that had formed from myoblasts dissociated from newborn rat hind-limbs during 9 days of culture. The cells were cocultured in Dulbecco's modified Eagle's medium, 5% horse serum, 100 μM hypoxanthine, 16 μM thymidine, and the following when indicated: Control, no addition; 1 mM theophylline; 10 μM PGE_1; 10 μM PGE_1 and 1 mM theophylline; or 1 mM dibutyryl cAMP. Synapses were detected by intracellular microelectrode recording of miniature end-plate potentials. Each point is the mean of values obtained from 10–20 myotubes. (Data from Higashida et al. 1978.)

Effects of Culture Conditions on ACh Storage and Secretion

As shown in Figure 3A, intracellular ACh levels of NG108-15 cells increased eightfold and threefold when cells were treated for 3 or more days with 10 μM PGE_1 and 1 mM theophylline or with 1 mM dibutyryl cAMP, respectively. The cells were incubated with 30 μM [^{3}H]choline for 17 hours to label intracellular ACh. Cells were then washed, and intracellular [^{3}H]ACh was extracted and quantitated. More than 90% of the intracellular [^{3}H]ACh was found to be particulate. Treatment of cells for 5 or more days with dibutyryl cAMP (Daniels and Hamprecht 1974) or with PGE_1 and theophylline (Wilson et al. 1978) resulted in marked increases in the abundance of small clear vesicles and large dense-core vesicles. These results suggest that the increase in intracellular ACh is due, at least in part, to

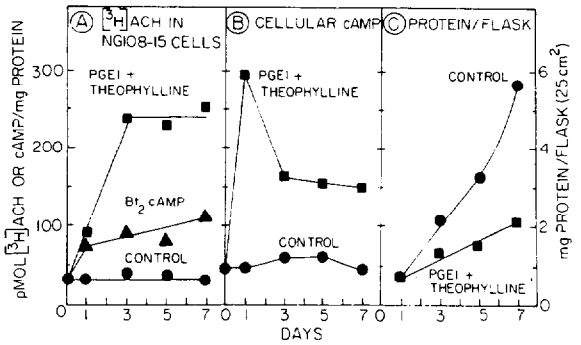

Figure 3. Effects of dibutyryl cAMP or PGE_1 and theophylline on intracellular [^{3}H]ACh levels of NG108-15 cells (*A*) and intracellular cAMP (*B*); (*C*) cell proteins per flask are shown as a function of time (Wilson et al. 1978). (*A*) NG108-15 cells were incubated in culture medium containing approximately 30 μM [*methyl*-^{3}H]choline chloride for 17 hr to enable cells to synthesize [^{3}H]ACh. Cells were washed 3 times with isotonic buffer, and then intracellular [^{3}H]ACh was extracted (Toru and Aprison 1966) and separated from other radioactive compounds by high-voltage paper electrophoresis (Potter and Murphy 1967). Where indicated, the medium was supplemented with the following: Control, no addition; 1 mM dibutyryl cAMP; or 10 μM PGE_1 and 1 mM theophylline. (*B*) cAMP was extracted from cells and purified as described by Matsuzawa and Nirenberg (1975); the amount of cAMP in each sample was determined by inhibition of the binding of [^{3}H]cAMP to cAMP-dependent protein kinase. (*C*) Protein was determined by a modification of the method of Lowry et al. (1951).

an increase in the number of ACh storage vesicles in cells.

In the presence of 10 μM PGE_1 and 1 mM theophylline, cAMP levels of NG108-15 cells increased markedly and then decreased somewhat during the remainder of the incubation period due to partial desensitization of PGE_1 receptors (Fig. 3B). However, cAMP levels of treated cells were higher than those of control cells throughout the 7-day period examined. The rate of cell division also decreased after the first day of treatment with PGE_1 and theophylline; thus, the rate of accumulation of protein per flask decreased in the presence of PGE_1 and theophylline (Fig. 3C).

The effect of treating NG108-15 cells for 0, 1, 3, or 5 days with 1 mM dibutyryl cAMP on the ability of cells to secrete ACh in response to a depolarizing stimulus is shown in Figure 4A. The cells were incubated with 27 μM [^{3}H]choline for 1 hour, washed by perfusion, and then depolarized with 80 mM K^+ (replacing 80 mM Na^+) during the period shown. [^{3}H]ACh secreted into the medium was separated from other ^{3}H-labeled compounds and quantitated as described by McGee et al. (1980). Logarithmically dividing, untreated control cells did not respond to depolarization by secreting ACh. However, cells treated 1, 3, or 5 days with 1 mM dibutyryl cAMP became increasingly responsive to depolarization with respect to the amount of ACh secreted (McGee et al. 1978). Cells treated with PGE_1 and theophylline for 7 days secreted twice as much ACh when depolarized as did cells that had been treated with dibutyryl cAMP (Fig. 4B).

Conditional Expression of Stimulus-Secretion Coupling in NBr10-A Cells

Depolarization of neuronal terminals is known to activate voltage-sensitive Ca^{++} channels; Ca^{++} ions then flow into the neuronal terminals and increase the rate of secretion of neurotransmitter. Thus, the effects of treating cells for 7 days with PGE_1 and theophylline or dibutyryl cAMP on Ca^{++} uptake via voltage-sensitive Ca^{++} channels were determined. $^{45}Ca^{++}$ ions rapidly bound to NBr10-A cells initially and/or entered cells incubated in medium containing 5.4 mM K^+ ions, but uptake soon plateaued. Depolarization of cells with 80 mM K^+ resulted in a marked increase in $^{45}Ca^{++}$ uptake. Half-maximal inhibition of depolarization-dependent uptake of $^{45}Ca^{++}$ was obtained with 9×10^{-7} M D600; La^{3+}, Co^{++}, or Ni^{++} ions also inhibited K^+-dependent $^{45}Ca^{++}$ by cells. Electrophysiological studies revealed Ca^{++} spikes; action potentials also were obtained when strontium or barium ions were substituted for Ca^{++} ions (not shown).

As shown in Figure 5, logarithmically dividing, untreated control NBr10-A cells lacked functional voltage-sensitive Ca^{++} channels and did not take up $^{45}Ca^{++}$ ions when cells were depolarized. However, cells treated for 7 days with 10 μM PGE_1 and 1 mM theophylline or with 1 mM dibutyryl cAMP took up $^{45}Ca^{++}$ via voltage-sensitive Ca^{++} channels when cells were depolarized with 80 mM K^+ (Rotter et al. 1979).

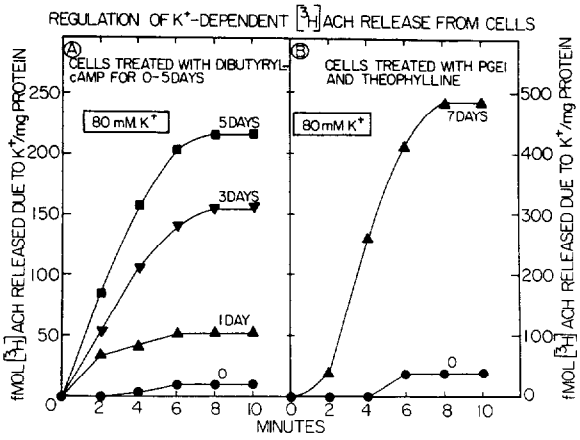

Figure 4. [^{3}H]ACh secretion by untreated control NG108-15 cells or by cells treated for 1, 3, or 5 days with 1 mM dibutyryl cAMP (*A*) or by cells cultured for 7 days with or without 10 μM PGE_1 and 1 mM theophylline (*B*) is shown in response to depolarization of cells with 80 mM K^+ (replacing 80 mM Na^+ in the medium). The cells were incubated with [*methyl*-^{3}H]choline chloride to enable them to synthesize [^{3}H]ACh, and then washed and depolarized. (Data from McGee et al. [1978]; the figure was redrawn.) [^{3}H]ACh was separated from other radioactive compounds and quantitated as described by McGee et al. (1980). The basal rate of [^{3}H]ACh secretion by unstimulated cells (5.4 mM K^+) was subtracted from each value shown.

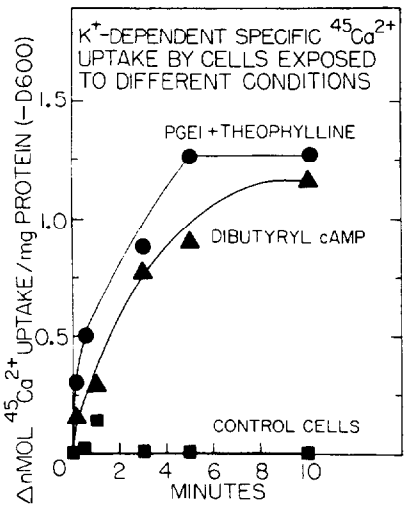

Figure 5. Effect of culture conditions on the expression of functional voltage-sensitive Ca^{++} channels of NBr10-A cells. $^{45}Ca^{++}$ uptake was due to activation of voltage-sensitive Ca^{++} channels of untreated, logarithmically dividing control NBr10-A cells and cells cultured for 6 days with 1 mM dibutyryl cAMP or 10 μM PGE_1 and 1 mM theophylline. The cells were depolarized with 80 mM K^+ (in place of 80 mM Na^+). Values for $^{45}Ca^{++}$ binding to cells and/or uptake at 5.4 mM K^+, which were not inhibited by 100 μM D600 and were not mediated by voltage-sensitive Ca^{++} channels were subtracted. Ca^{++} uptake dependent on depolarization was completely inhibited by 100 μM D600. (Data from Rotter et al. 1979)

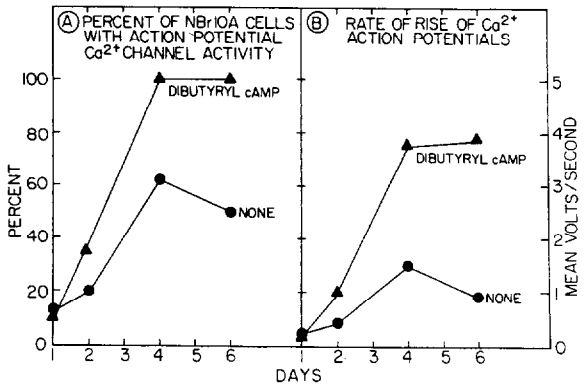

Figure 6. Acquisition of functional voltage-sensitive Ca^{++} channels by NBr10-A cells cultured with or without 1 mM dibutyryl cAMP is shown as a function of time of treatment. Ca^{++} action potentials were detected by intracellular microelectrode recording; each point is the mean of values obtained from 20 NBr10-A cells. Logarithmically dividing NBr10-A cells reached confluency on approximately the fourth day of culture. The medium contained 5 μM tetrodotoxin to inhibit voltage-sensitive Na^+ channels. (Data from Rotter et al. 1979.)

Four methods were used to measure Ca^{++} uptake dependent on cell depolarization: $^{45}Ca^{++}$ flux was measured; net uptake of Ca^{++} ions from the medium by cells was determined using a Ca^{++}-specific electrode; Ca^{++} ion concentrations were determined by a spectrophotometric assay with murexide (P. Darvenezia and M. Nirenberg, in prep.); and Ca^{++} action potentials were demonstrated by electrophysiological methods with intracellular microelectrode recording. Results obtained by each method showed that the expression of voltage-sensitive Ca^{++} channels is increased by treatment of cells with compounds that elevate cellular cAMP levels.

The acquisition of voltage-sensitive Ca^{++} channel activity by NBr10-A cells measured with intracellular microelectrodes is shown in Figure 6 as a function of days of culture of cells with or without 1 mM dibutyryl cAMP. Cells were stimulated electrically with depolarizing pulses of current in the presence of 5 μM tetrodotoxin to inhibit voltage-sensitive Na^+ channels. Voltage-sensitive Ca^{++} channel activity was determined at the resting membrane potential and at a membrane potential of −90 mV adjusted with steady current. Only 10% of logarithmically dividing, untreated cells at low density possessed functional voltage-sensitive Ca^{++} channels, and the Ca^{++} spikes that were detected were relatively weak. The proportion of cells with Ca^{++} spikes increased from 10% to 50% as untreated cells multiplied and formed confluent monolayers, whereas 100% of the cells tested that were treated for 3 days with dibutyryl cAMP generated Ca^{++} spikes when stimulated electrically. The mean maximum rate of rise of Ca^{++} spikes, a measure of Ca^{++} channel activity, increased approximately 20-fold when cells were treated with dibutyryl cAMP (M. Adler and M. Nirenberg, in prep.) (Fig. 6B). A smaller increase was observed when logarithmically dividing, untreated control cells formed confluent monolayers, revealing a small cell concentration or contact-dependent regulation of Ca^{++} channel expression. These and the previous results show that when cAMP levels are elevated, cells acquire at similar rates voltage-sensitive Ca^{++} channels, depolarization-dependent ACh secretion, and synapses.

Nitrendipine and other dihydropyridines have been shown to reduce voltage-sensitive Ca^{++} channel activity in smooth muscle (for review, see Triggle and Swamy 1983), and specific binding sites for [^{3}H]nitrendipine have been found in smooth muscle (Bolger et al. 1982), cardiac muscle (Ehlert et al. 1982), and brain (Murphy and Snyder 1982). The receptors for nitrendipine are thought to be either part of the voltage-sensitive Ca^{++} channel complex or regulators of channel activity. As shown in Table 3, few or no specific binding sites for [^{3}H]nitrendipine were detected in washed membranes prepared from logarithmically dividing control NBr10-A cells, whereas specific binding sites were found in membranes prepared from NBr10-A cells that had been treated for 8 days with 10 μM PGE_1 and 1 mM theophylline (M. Nirenberg, L. Anderson, and A. Rotter, in prep.). Scatchard analysis revealed a single class of specific binding sites for [^{3}H]nitrendipine with a dissociation constant of 2×10^{-10} M, which agrees well with values reported for other tissues (Bolger et al. 1982; Murphy and Snyder 1982). The maximum number of specific nitrendipine-binding sites in membranes from NBr10-A cells treated with PGE_1 and theophylline was found to be 61 fmoles/mg of membrane protein, which corresponds to approximately 16,000 sites for nitrendipine per cell. SB37-B neuroblastoma × L cell hybrid cells synthesize ACh but have little or no voltage-sensitive Ca^{++} channel activity and thus do not secrete ACh when cells are depolarized and do not form synapses with muscle cells. Specific binding sites for [^{3}H]nitrendipine were not detected in membranes prepared from SB37-B cells grown with or without PGE_1 and theophylline. These results show that the cAMP-dependent expression of

Table 3. PGE_1 and Theophylline Regulate the Number of Nitrendipine-binding Sites Expressed by NBr10-A Cells

Treatment of cells (5–8 days)	fmoles [^{3}H]nitrendipine specifically bound/mg protein
NBr10-A	
control	<2
10 μM PGE_1 + 1 mM theophylline	17
SB37-B	
control	<2
10 μM PGE_1 + 1 mM theophylline	<2

Data from M. Nirenberg, L. Anderson, R. Rotter, and R. Ray (in prep.). The reaction-mixture components and conditions were similar to those reported by Murphy and Snyder (1982). Each reaction mixture contained 0.2 nM [^{3}H]nitrendipine and approximately 250 μg of protein (a washed membrane fraction pelleted at 48,000*g*).

voltage-sensitive Ca^{++} channels in NBr10-A cells is accompanied by an increase in the number of specific binding sites for [^{3}H]nitrendipine and suggest that elevation of cellular cAMP results in an increase in the number of Ca^{++} channels in cells.

cAMP-dependent Changes in Glycoproteins of NG108-15 Cells

Elevation of cAMP levels of neuroblastoma or hybrid cells gradually results in the acquisition by cells of functional action potential channels for Na^+, K^+ (Nelson et al. 1978), and Ca^{++} (Rotter et al. 1979), which suggests that cAMP regulates the synthesis or catabolism of channel molecules. We therefore determined the effect of PGE_1 on glycoproteins expressed by NG108-15 cells. Logarithmically dividing control NG108-15 cells and cells treated with 10 μM PGE_1 for 7 days were incubated for 18 hours with [^{35}S]methionine. ^{35}S-labeled glycoproteins soluble in 1% Triton X-100 were fractionated by chromatography on wheat germ agglutinin-, *Lens culinaris* agglutinin-, and *Ricinus communis* agglutinin$_{II}$-agarose columns. ^{35}S-labeled glycoproteins were eluted with 0.5 M *N*-acetylglucosamine, 0.2 M α-methylmannoside, and 0.2 M lactose, respectively, desalted, and subjected to two-dimensional polyacrylamide gel electrophoresis and autoradiography (Fig. 7). Autoradiographs of duplicate two-dimensional gels (triplicates in other experiments), exposed for 7 hours and 1, 2, and 4 days, were compared with the aid of a computer and programs designed for analysis of two-dimensional gels (Vo et al. 1981). Computer-generated plots based on the 48 autoradiographs analyzed in this experiment were obtained.

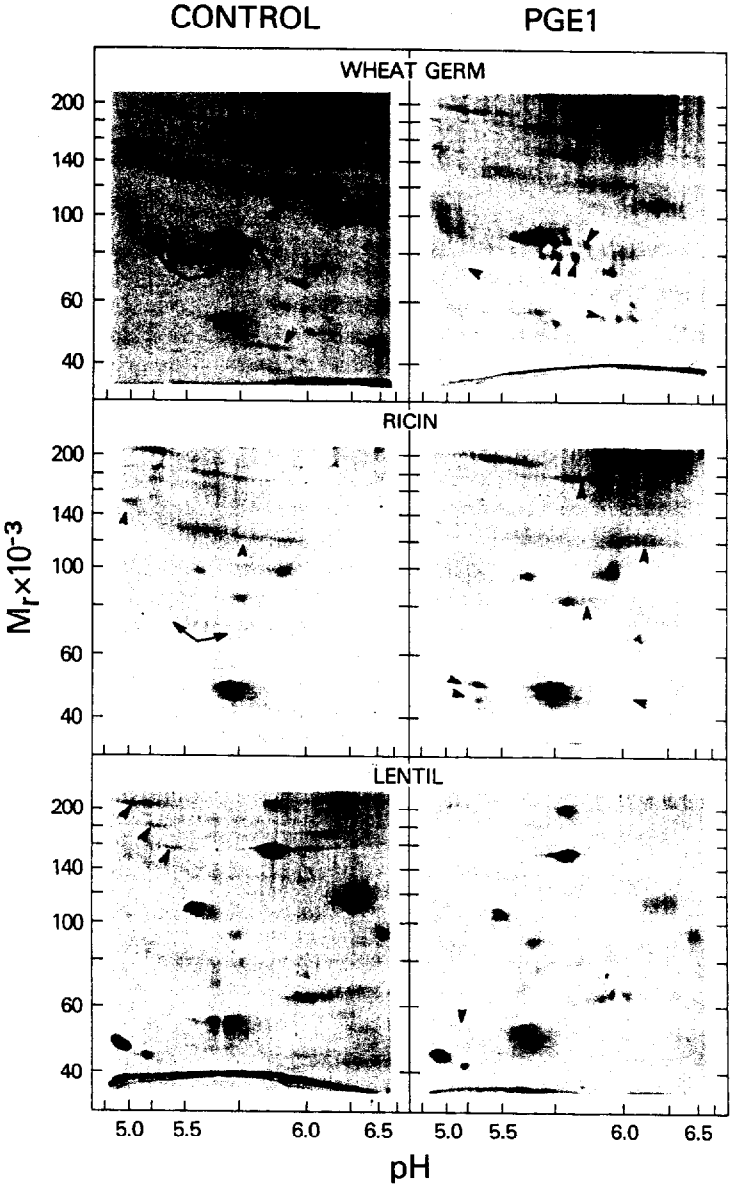

Figure 7. Two-dimensional electrophoresis of ^{35}S-labeled glycoprotein fractions. NG108-15 cells treated for 6 days with 10 μM PGE_1 and logarithmically dividing, untreated control cells were incubated for 18 hr in Dulbecco's modified Eagle's medium that contained 2% fetal bovine serum and 5 μM L-[^{35}S]methionine (100 μCi/ml of medium). The cells were harvested and washed in isotonic phosphate-buffered saline. The pelleted cells were then lysed, and the proteins were solubilized by the addition of a solution containing 10 mM HEPES (pH 7.4); 150 mM NaCl; 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$; 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Cell lysates were centrifuged at 100,000*g* for 1 hr at 3°C, and glycoproteins in the supernatant portions were fractionated by wheat germ agglutinin-, *L. culinaris* agglutinin-, and *R. communis* agglutinin$_{II}$-agarose column chromatography. ^{35}S-labeled glycoproteins were eluted with 0.5 M *N*-acetylglucosamine, 0.2 M α-methylmannoside, or 0.2 M lactose, respectively, dialyzed against 0.01 M NH_4HCO_3, and lyophilized. Two portions from each sample were subjected to two-dimensional polyacrylamide gel electrophoresis on duplicate 7.5% acrylamide gels according to the method of O'Farrell (1975) with 1.0×10^6 cpm (10–20 μg of protein) applied to each gel. Autoradiographs were exposed for 7 hr and 1, 2, and 4 days; those shown were exposed for 2 days. Arrows indicate some of the cAMP-dependent differences in ^{35}S-labeled glycoproteins, i.e., control cells vs. cells treated with PGE_1. (Data from K.E. Krueger, M.I. Miller, and M. Nirenberg, in prep.)

In most cases, different species of ^{35}S-labeled glycoproteins were eluted from the three lectin columns. Most species of ^{35}S-labeled glycoproteins were synthesized by both control cells and PGE_1-treated cells. However, numerous cAMP-dependent changes in the ^{35}S-labeled glycoproteins were found as summarized in Table 4. Twelve ^{35}S-labeled glycoproteins were expressed by PGE_1-treated cells that were not detected in extracts of logarithmically dividing control cells. In addition, the radioactivities of 29 ^{35}S-labeled glycoproteins expressed by cells treated with PGE_1 were 2.5- to 10-fold higher than those of control cells. cAMP-dependent decreases in the ^{35}S-labeled glycoproteins were also found. Forty-eight ^{35}S-labeled glycoproteins were expressed by control cells but not by cells treated with PGE_1. In addition, the radioactivities of 25 ^{35}S-labeled glycoproteins expressed by control cells were 2.5–6.5-fold higher than those of cells treated with PGE_1. No changes in the ^{35}S-labeled glycoproteins were detected when cells were incubated with 10 μM PGE_1 for 20 minutes (not shown here).

DISCUSSION

Neuroblastoma hybrid cells were obtained that synthesize ACh and form synapses with cultured striated muscle cells. Other cell lines were found that synthesize ACh, adhere to myotubes, but form few or no synapses. Different kinds of presynaptic defects were identified, such as cell lines without voltage-sensitive Ca^{++} channel activity, large dense-core vesicles, depolarization-dependent ACh secretion, or active protein that increases the aggregation of nicotinic AChR on myotube plasma membranes.

No immediate effect of cAMP was detected on voltage-sensitive Ca^{++} channel activity, ACh secretion, or the percentage of myotubes that were innervated. In contrast, these properties and synaptogenesis were regulated by prolonged elevation of cAMP levels of hybrid cells. Activation of adenylate cyclase with 10 μM PGE_1 for 5 days, or treatment of cells with 1 mM dibutyryl cAMP, resulted in 10–100-fold increases in voltage-sensitive Ca^{++} channel activity, 15–45-fold increases in spontaneous secretion of ACh at synapses, and 5–10-fold increases in the number of muscle cells innervated. Treated cells also contained considerably more large dense-core vesicles and small clear vesicles and had higher levels of intracellular ACh than did untreated cells.

Treatment of NBr10-A cells with 10 μM PGE_1 and 1 mM theophylline resulted in the appearance of specific binding sites for [^{3}H]nitrendipine, a putative antagonist of voltage-sensitive Ca^{++} channels. Specific nitrendipine-binding sites were not detected in SB37-B cells, which lack voltage-sensitive Ca^{++} channel activity. These results suggest that cells acquire more voltage-sensitive Ca^{++} channels when cellular cAMP is elevated for a number of days.

By regulating the expression of Ca^{++} channels in hybrid cells, cAMP would be expected to function as a conditional switch that modulates the activities of many Ca^{++}-dependent reactions in the neural cells and couples these reactions to the cell membrane potential and to transynaptic or hormonal stimuli that affect adenylate cyclase activity.

Glycoproteins of NG108-15 hybrid cells grown in the presence of [^{35}S]methionine with or without 10 μM PGE_1 were solubilized and fractionated by wheat germ agglutinin, lentil lectin, or ricin column chromatography and by two-dimensional gel electrophoresis. Elevation of cellular cAMP levels resulted in the disappearance of some glycoproteins, the appearance of new glycoproteins of different relative molecular weights, large increases or decreases in the radioactivities of some ^{35}S-labeled glycoproteins, and changes in the isoelectric points of still other ^{35}S-labeled glycoproteins. These results agree with and extend previous reports of differentiation-specific changes in neuroblastoma proteins or glycoproteins (Truding et al. 1974, 1975; Charalampous 1977; Garvican and Brown 1977; Prashad et al. 1977; Akeson and Hsu 1978; Prashad and Rosenberg 1978; Rosenberg et al. 1978; Zisapel and Littauer 1979; Littauer et al. 1980; Atkinson and Bramwell 1981; Sugiyama et al. 1980; Imada and Imada 1982).

Treatment of neuroblastoma or hybrid cells with dibutyryl cAMP has been shown to alter the levels of some species of polysomal mRNA (Morrison et al. 1980; U.Z. Littauer, pers. comm.). When "undifferentiated" neuroblastoma cells were shifted to a more differentiated state by altering various growth conditions, some species of polysomal poly(A)$^+$ RNA were no longer expressed (Felsani et al. 1978; Grouse et al.

Table 4. cAMP-dependent Changes in ^{35}S-labeled Glycoproteins

Effects of cAMP on ^{35}S-labeled glycoproteins	No. of ^{35}S-labeled glycoproteins: control cells	No. of ^{35}S-labeled glycoproteins: PGE_1 cells
Increased expression	0	12
Increased expression	29	29↑
Decreased expression	48	0
Decreased expression	25	25↓

The experiment is described in Fig. 7 (K.E. Krueger, M. Miller, and M. Nirenberg in prep.). The total number of ^{35}S-labeled proteins detected in extracts of control cells was 317. Twelve additional ^{35}S-labeled proteins were detected in extracts of cells that had been treated with PGE_1.

1980) and many new species of poly(A)$^+$ RNA appeared (Grouse et al. 1980). Marked effects of cAMP or dibutyryl cAMP on mRNA levels of other eukaryotic cells have been reported (Derda et al. 1980; Maurer 1981; Miles et al. 1981; Lamers et al. 1982; Landfear et al. 1982; Wu and Johnson 1982; Mangiarotti et al. 1983).

Twelve new ^{35}S-labeled glycoproteins were expressed by cells treated with PGE_1, and 2.5–10-fold increases were found in the radioactivities of 29 additional ^{35}S-labeled glycoproteins. Concomitantly, neuroblastoma or hybrid cells acquired voltage-sensitive Na^+ (Catterall et al. 1973), K^+ (Nelson et al. 1969), and Ca^{++} channels; Ca^{++}-dependent K^+ channels; extended long neurites; and acetylcholinesterase specific activity increased (Blume et al. 1970). These results suggest that some of the ^{35}S-labeled glycoproteins that are expressed to a greater extent in PGE_1-treated cells may play a role in some of the newly expressed neuronal membrane functions. Synapse-defective cell lines that lack channel activity or the ability to extend neurites and other variant cell lines may provide a means of identifying functions of some membrane proteins.

In summary, the results show that cAMP regulates synaptogenesis by regulating the expression of voltage-sensitive Ca^{++} channels and suggest that cAMP affects posttranslational modifications of some species of glycoproteins and/or regulates gene expression.

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